WYATT TECHNOLOGY

White Paper

miniDAWN TREOS II
The Next Generation Compact SEC-MALS Detector

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Introduction

Online multi-angle light scattering (MALS) detectors measure the light scattering properties of polymers, proteins, peptides and nanoparticles, in conjunction with SEC, in order to characterize essential physiochemical properties: molar mass, size, and conformation. Binary conjugates such as glycoproteins and copolymers, which are not amenable to standard SEC analysis, may also be characterized by SEC-MALS. The miniDAWN® TREOS® II, heralding the next generation of compact online MALS instruments, incorporates multiple technological innovations that enhance performance and productivity in SEC-MALS and other MALS applications such as FFF-MALS and CG-MALS. This paper describes those innovations and the benefits accrued as a result of their implementation.

MALS explained

Online MALS has long been used with gel permeation chromatography (GPC) or size exclusion chromatography (SEC) for the absolute characterization of macromolecules and nanoparticles [1]. A sensitive MALS detector utilizes a low-stray-light flow cell, monochromatic laser illumination and a high-gain optical read head, illustrated schematically in Figure 1, to measure the angle-dependent Rayleigh ratio R(θ) (i.e. the ratio of laser intensity scattered by the analyte relative to the intensity scattered by the solvent, for each angle θ at which a detector is placed).

The values of R(θ) are analyzed along with the concentration—determined simultaneously by an online concentration detector such as the Optilab® T-rEX differential refractive index (dRI) detector or a UV/Vis absorbance detector—to calculate the molecular weight of the sample MW, and its root-mean-square (rms) radius r_g. For some samples, both a dRI and a UV detector are utilized in tandem. A complete SEC-MALS system is depicted in Figure 2.

This measurement is performed for each volume of solution eluting from the chromatography column and passing through the flow cell in order to calculate rigorous distributions of MW and r_g. MALS provides a first-principles analysis of molecular weight and size, independent of reference standards or assumptions of ideal SEC/GPC column retention. Hence these results are not affected by molecular conformation, chemical properties or the availability of appropriate reference molecules. They are considered 'absolute' and far more reliable than standard SEC.

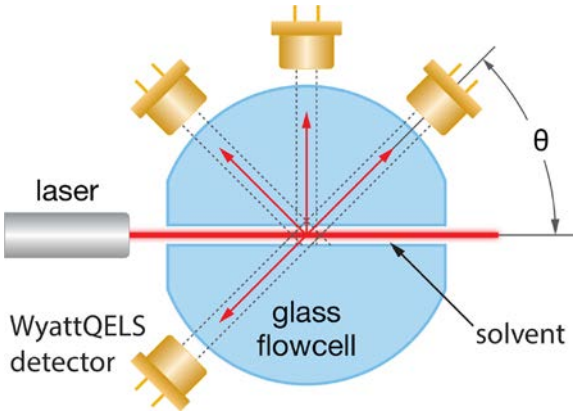

Figure 1. MALS measurement diagram. The co-aligned laser beam and solution paths are instrumental in minimizing stray light for maximum sensitivity. Detection at three optimally-positioned scattering angles enables measurement of size from 10 to 50 nm in rms radius. The WyattQELS DLS detector extends size measurements down to 0.5 nm in hydrodynamic radius.

The molar mass range that may be determined by a premier MALS detector such as the DAWN® HELEOS® II is 200 Da – 1 GDa, and the size range for root mean square (rms) radius is 10 nm – 500 nm. A compact MALS detector such as the miniDAWN covers a smaller range, 200 Da - 10 MDa in molar mass and 10 nm - 50 nm in r_g (extending to 150 nm for particles with well-defined shapes such as sphere or rod) which is appropriate and cost-effective for a wide variety of peptides, proteins and smaller polymers and nanoparticles.

While MALS readily measures MW for molecules that are below 10 nm in r_g, it cannot measure their size. The addition of an embedded WyattQELS dynamic light scattering module to the MALS detector provides the capability of measuring size down to 0.5 nm in hydrodynamic radius.

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Figure 2. SEC-MALS setup. The miniDAWN and Optilab interface to standard HPLC systems for absolute molar mass, size and conformation.

Overcoming the limits of SEC

Figure 3 and Figure 4 illustrate how single-detector GPC based on column calibration leads to erroneous molecular weight results, while MALS results are robust and accurate. For polymers, SEC-MALS analysis can yield essential physiochemical properties, including

- Conformation (random coil, branched, globular)
- Mark-Houwink-Sakurada parameters
- Branching ratio, branches per molecule and drainage coefficient [2]

In addition to characterizing typical proteins or polymers via basic *dual-detection* SEC-MALS, binary complexes—not otherwise amenable to SEC characterization—may be characterized by means of a *triple-detection* SEC-MALS-UV-dRI setup [3]. These species include

- glycoproteins
- protein-polysaccharide conjugate vaccines
- PEGylated proteins
- surfactant-solubilized membrane proteins
- copolymers.

With an extensive install base, and thousands of peer-reviewed publications citing data obtained with the miniDAWN, this instrument is widely accepted and has proven to be essential for laboratories performing macromolecular characterization or otherwise engaged in producing and validating proteins and small polymers.

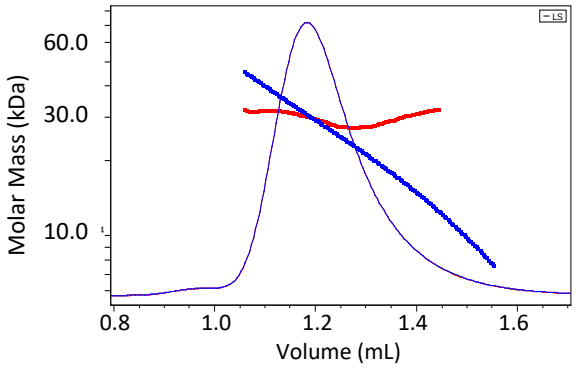


Figure 3. Comparison of single-detector GPC (blue) and MALS analysis (red) of molecular weight for a 29 kDa polystyrene standard. While MALS shows a narrow molecular weight range around 29-30 kDa, column calibration *by definition* implies (incorrectly) that the peak is polydisperse from 10-50 kDa.

What's missing?

The utility of any analytical instrument is a function of its performance and day-to-day productivity. While the previous model of compact MALS detector, the miniDAWN TREOS, outstripped the competition in terms of performance and productivity, an analysis of industry trends and customer needs determined that certain key aspects of the instrument should be improved. The miniDAWN TREOS II was designed to incorporate these features, continuing to provide the solution for absolute macromolecular characterization well into the future and keeping Wyatt's customers delighted.

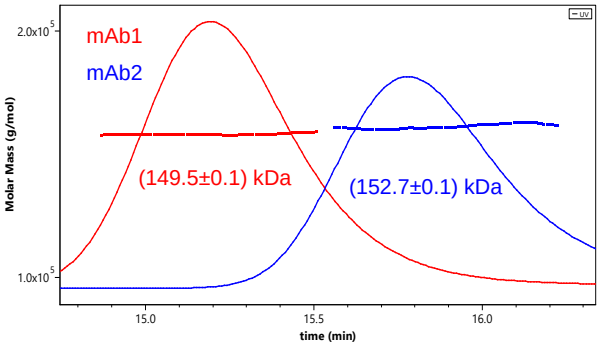


Figure 4. Elution of two monoclonal antibodies. Despite the different elution times, these molecules have essentially the same molecular weight,shown to be true by MALS.

Building an *even better* MALS detector

Improvements to the miniDAWN were tackled on two fronts: performance and productivity. The latter includes several new features that guarantee users can make more measurements, with more up time, more convenience and a built-in upgrade path to cutting-edge UHPLC instrumentation.

Performance

MALS: a sensitivity boost

The three-detector MALS geometry of the miniDAWN depicted in Figure 1, along with the laser wavelength, dictate the range of sizes that can be measured by MALS. These have been optimized from the design of the original miniDAWN MD30 (introduced in the year 2000) to provide accurate measurements up to 50 nm in r_g. This range covers most proteins, small polymers and basic macromolecular assemblies such as virus-like particles. The maximum molecular weight that can be measured is actually limited by the upper limit of r_g and the molecule's conformation: compact molecules such as globular proteins or highly branched polymers can be measured accurately up to 10 MDa while extended molecules such as linear polymers or denatured proteins can only be measured up to 1 MDa. In order to keep the miniDAWN a low-cost, but high-quality, compact MALS detector for its target applications, the fundamental read head design was maintained at three angles.

However, a key performance parameter that can *always* be improved upon—and should be!—is sensitivity. Higher sensitivity translates into lower sample consumption as well as detection and characterization of smaller amounts of trace peaks such as aggregates, fragments or other impurities.

In a well-designed optical system free of stray light, such as that engineered into the miniDAWN, sensitivity can be increased by simply increasing the laser intensity. Thanks to advances in commercial laser technology, new, low-cost lasers are available which are fully compatible with the miniDAWN TREOS electro-optical design yet provide 50% higher intensity: the power was increased from 50 mW to 75 mW. Since sensitivity is generally proportional to the square root of laser intensity, this power boost provides a corresponding ~20% increase in sensitivity: from 30 ng of 100 kDa polystyrene in THF (assuming injection on a standard 7.8 mm i.d. GPC column) to just 25 ng. This level of sensitivity positions the TREOS II at 4x the sensitivity of its closest competitor.

DLS: range farther

The primary purpose of the WyattQELS embedded DLS module is to measure particle size below the 10-nm cutoff of MALS, down to 0.5 nm in hydrodynamic radius. There are, however, good reasons to make use of DLS for larger sizes:

- Due to the large imaginary component in their refractive indices, MALS does not provide a good solution to size measurement of metallic nanoparticles and semiconducting quantum dots. DLS does an excellent job of making size measurements on these particles.
- The *combination* of MALS and DLS provides an indication of shape and structure for nanoparticles with more ordinary refractive indices.

Given the importance of performing DLS measurements, the upper range that can be accurately quantified is no less important than the lower limit. The miniDAWN TREOS had an upper limit of 40 nm. We decided to stretch the range even farther in the TREOS II, by shifting the detection angle from 90° to 135°. The new detection angle increases the total r_h range by 25%: it now covers 0.5 – 50 nm at a typical SEC flow rate of 0.5 mL/min, matching the upper limit to that of rms radius measurement by MALS, with no cost in terms of DLS sensitivity. The TREOS II can analyze a larger range of Au and Ag nanoparticles as well as the conformation and structure of VLPs, vesicles and other nanoparticles.

Productivity

The TREOS II offers productivity gains on several fronts, including ease-of-use, field serviceability and upgradeability to UHPLC.

Ease of use

For many years, SEC-MALS was considered cutting-edge technology intended mostly for experts such as academics and Ph.D.-level R&D scientists. The widespread adoption of light scattering into quality control and other routine GPC analyses was impeded, to a large degree, by the perception that the measurement and analysis of protein or polymer

samples by SEC-MALS is not for "mere mortals". The TREOS II implements several new features that make SEC-MALS measurements and analyses routine without compromising on rigor or the ability of experts to delve into the details of the analysis.

How it's been done

SEC-MALS requires very clean mobile phase, often achieved only after equilibrating the SEC column extensively and sometimes necessitating a new filter membrane on the pump filter. The first obstacle to SEC-MALS analysis was determining if the cleanliness of the mobile phase was suitable for a good measurement. The only way to estimate the cleanliness level was to eyeball the voltage noise on the instrument front panel, to know what the appropriate level is for the specific instrument, and compare the two.

After completing the measurement, data analysis required many steps such as selecting the appropriate analysis method for the specific sample type (e.g. protein, conjugated protein or polymer), setting the normalization constants, setting baseline, setting the alignment and band broadening correction between MALS and concentration signals, selecting the peak region in the chromatogram, entering sample and solvent parameters, verifying which (if any) of the MALS detectors should be ignored because of noisy chromatography, identifying the right order of angular dependence…. clearly a daunting task to complete for non-experts.

In the TREOS II

The TREOS II provides a direct, specific indication to the user regarding solvent noise levels: its internal computer measures baseline noise continuously, compares it to the appropriate acceptable noise levels, and sets the color of the 'System Ready' light on the front panel graphic display red, yellow or green accordingly. The System Ready indicator applies a smart algorithm that ignores any chromatographic peaks which might come through, and takes the burden off the user of assessing the noise level. If the noise does not decrease after flushing the column extensively, the user can take action such as changing the filter membrane.

Perhaps more significantly for the user, the ASTRA chromatography software that collects and analyzes MALS data from the miniDAWN TREOS II has received a major makeover in terms of ease of use. ASTRA versions 7.1 and higher, included with the purchase of a TREOS II, implement two major productivity enhancements for routine SEC-MALS use: Method Builder Wizard and One-Click Mw™.

- **Method Builder Wizard:** ASTRA offers many analyses, running the gamut from simple molar mass determination to complex calculations of cumulative and differential distributions, branching ratios, particle shape factor and protein conjugates. It is not always clear to non-expert users which analysis is appropriate per sample type and which parameters must be entered by the user. The new Method Builder Wizard allows for the creation of a default method that is automatically adapted to the correct sample type and instruments present.

Figures 4 - 6 present the three brief steps involved in setting up the default method: specifying the type of sample, the HPLC and detectors, and the relevant input parameters needed and selection between manual or automatic data processing.

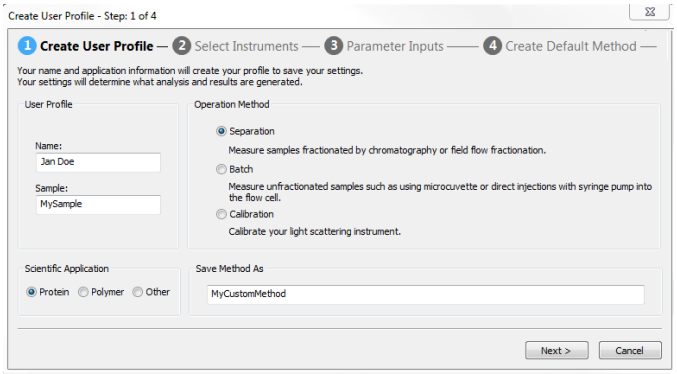


Figure 5. Method Builder Wizard step 1

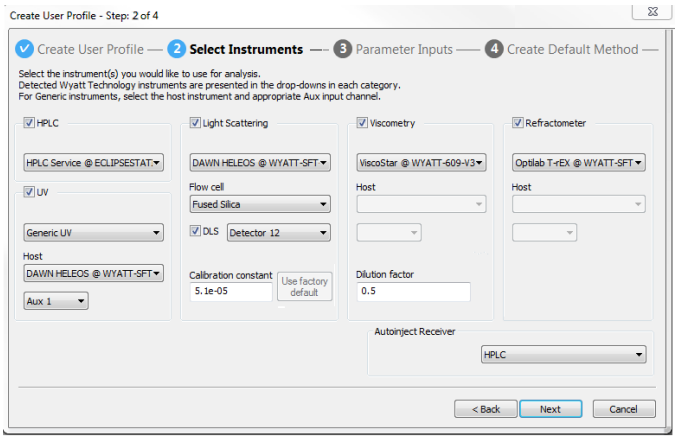
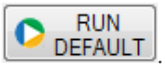


Figure 6. Method Builder Wizard step 2.

- **One-Click Mw:** Once saved, the default method can then be run at the click of a single button that appears on the main screen of ASTRA:



If Automatic Data Processing has been selected ASTRA will automatically set baselines and select all peaks in the chromatogram for analysis, complete the analyses that have been predefined and produce a report. This frees

the user from running through dozens of steps, making routine analyses nearly effortless.

However, One-Click Mw is most certainly *not* a black box! In accord with Wyatt's philosophy of scientific rigor, ASTRA *always* allows the user to go into the processing steps, verify that automatic processing completed by the program makes sense, the curves fit the data points, and if necessary make adjustments.

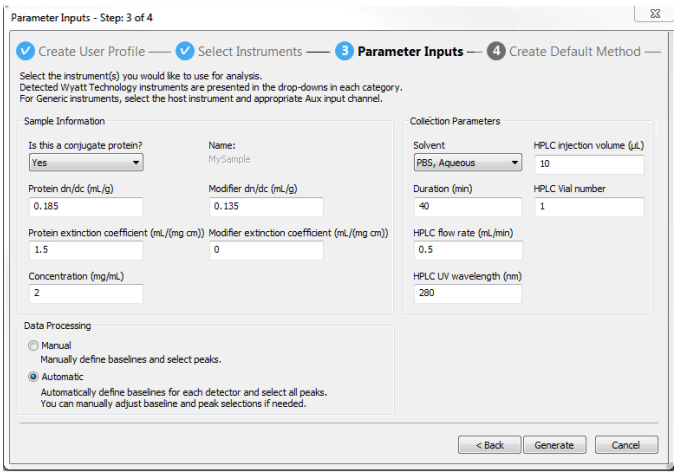


Figure 7. Method Builder Wizard step 3

Field Serviceability

As well designed as the TREOS II is, it is impossible to guarantee that a failure will never occur. When a MALS detector is dedicated to a mission-critical task such as lot release QC, any downtime translates into monetary loss. Bringing the detector back into service ASAP is essential.

How it's been done

On other instruments, some failures can be diagnosed and fixed in the field, but most significant issues require shipping back to the factory for proper service. This could translate into reams of paperwork, weeks of lost operation, and upon the instrument's return, additional time and resources dedicated to re-validation and paperwork.

In the TREOS II

The TREOS II is constructed of multiple, independent modules which are readily interrogated, diagnosed and—if necessary—replaced at the customer site by a field service engineer.

CheckPlus

The first step in maintenance and repair is diagnosis. However, it's not always easy to obtain an on-site expert diagnosis in short order. The TREOS II logs a slew of information that can be mined to determine its health and potential faults, but how to get the data to an engineer? That's why Wyatt created CheckPlus™, a desktop application that (upon initiation by the user) collects information and event logs from the TREOS II's modules and on-board computer, packs it up and e-mails it to a service engineer at Wyatt headquarters where the data can be analyzed and investigated for any issues.

If it looks like a specific module may be in need of repair, a service technician can run a full internal test suite from CheckPlus that assesses all the functions of the primary electronics boards.

Module swapping

Each of the primary modules is independently calibrated at the factory, so in case of failure, all the technician needs to do is swap in a new one. This applies to the power module, the analog circuit module, digital circuit module, the computer and display. Even the optical module—which includes the laser, detectors and flow cell—arrives fully aligned and calibrated, with its own event logging, and is a drop-in replacement for the old optical module.

Organizations that operate multiple TREOS II instruments in mission-critical tasks will find it cost-effective to keep on hand at least one backup for each module. Then time-to-repair is not much longer than the time it takes for a field service engineer to arrive on site, and much paperwork and revalidation work associated with sending an instrument off-site can be avoided.

Upgradeability to UHPLC

Ultra-high performance liquid chromatography (UHPLC) offers faster separations, higher resolution, and reduced consumption of sample and mobile phase than standard HPLC. It is being adopted across many industry and research labs where those benefits justify the additional cost. However, a great deal of resources has been invested in developing and validating robust HPLC methods, and it may not be worth abandoning these, especially for commercial products produced under a GMP regulatory regimen. For this reason, many acquisitions of new LC equipment consist of UHPLC-capable instrumentation that is operated initially with standard HPLC columns per well-established and validated HPLC methods. When new products—for which GMP HPLC methods have not yet been developed—are brought into the analytical lab, they will be characterized with newly-developed UHPLC methods.

MALS detectors, including the previous generation miniDAWN TREOS, are incompatible with UHPLC-SEC due to a large flow cell that introduces excessive dispersion, losing resolution and sensitivity. Another solution is needed for MALS measurements with UHPLC.

How it's been done

Wyatt Technology offers a MALS detector for UHPLC-SEC, the µDAWN™. With a µDAWN, all the benefits of SEC-MALS—absolute molar mass and size, conformation, conjugate analysis, etc.—may be transferred to UHPLC for faster throughout, better resolution and lower material consumption. In addition to UHPLC, the µDAWN may be operated with standard HPLC by means of an adapter kit for additional versatility. The µDAWN is, at the time of this writing, the only MALS detector for UHPLC yet maintains the high standards of all of Wyatt's MALS instruments.

In the TREOS II

Customers who are currently running HPLC methods and are considering transitioning to UHPLC but do not know exactly if or when this will occur may not be ready to invest in a µDAWN, which is significantly more costly than the miniDAWN TREOS II. In order to assist these customers, Wyatt has leveraged the modularity of the TREOS II: the instrument incorporates a built-in upgrade pathway to UHPLC-SEC. A field service engineer can simply swap out, at the customer site, the standard optical module for the µDAWN optical module which arrives fully aligned and calibrated. A few screws and electrical connections, and it is ready to go!

With a few minor operations—updating the on-board computer's firmware, changing the inlet and outlet fittings and replacing the front panel door labeled 'TREOS II' with one that sports the 'µDAWN' label—the instrument is now a fully-fledged µDAWN. Since the upgrade cost includes the HPLC adapter kit, the user can still revert to HPLC methods as needed while gaining all the benefits of UHPLC-SEC.

Productivity: the Bottom Line

The miniDAWN TREOS II ensures that users will be more productive, now and in the future, with:

- routine, easy operation and data analysis;
- maximum uptime;
- upgradeability to UHPLC-SEC

The next generation of compact MALS

The miniDAWN TREOS was a dependable and accurate compact MALS detector that outperformed all competing instruments. Where the additional capabilities and superior sensitivity of the DAWN HELEOS II were not needed, the TREOS brought the benefits of absolute macromolecular characterization to hundreds of protein, polymer and nanoparticle analytical labs. With the newest generation of miniDAWN, the TREOS II, we set out to make SEC-MALS even more accessible so that routine users would feel enabled and comfortable relying on this technology for high-value-added work.

Validating sensitivity

The new instrument's enhanced sensitivity was validated via a series of injections of 105 kDa polystyrene, shown in Figure 7. The specification for accuracy in molar mass of ±5% was maintained down to just 25 ng total injected mass, and exceeded at 10 ng. For comparison, the original TREOS exhibited a lower limit of 30 ng using the same protocol.

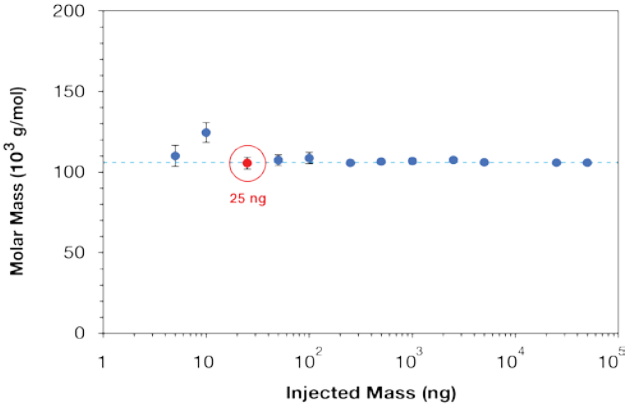


Figure 8. Molar masses determined by the TREOS II for a series of injections of 105 kDa polystyrene in THF, using standard GPC, with decreasing total injected mass. Excellent and consistant results are maintained through 25 ng injections.

We note that the previous sensitivity specification for the miniDAWN was based on BSA in PBS. However, since sensitivity in aqueous chromatography tends to be limited by column shedding and other sources of external noise rather than the instrument itself, it was decided that polystyrene in THF would be a more representative specification for ultimate sensitivity. Anyone performing aqueous chromatography can usually expect lower sensitivity than organic chromatography. Still, with a moderate degree of effort, the TREOS II can accurately characterize less than 1 µg of BSA in PBS and less than 400 ng of IgG.

The upgrade really works!

We know that UHPLC holds the promise for faster runs, better resolution and lower sample consumption… but does the TREOS II upgrade really work as promised? To validate the upgrade, we tested an injection of monoclonal antibody on the as-provided TREOS II using HPLC (Wyatt SEC column, 100 Å pores, 5 µm beads, 7.8 mm x 300 mm, 200 µg injected mass), and on the upgraded instrument using UHPLC (Waters UPLC BEH SEC column, 200 Å pores, 1.7 µm beads, 4.6 mm x 300 mm, 30 µg injected mass).

The results, shown in Figure 8, clearly convey the resolution advantage of UHPLC: the main (monomer) peak is much narrower, even on a relative column volume basis, and the aggregates better resolved. The UHPLC chromatogram exhibits a fragment peak that is not distinguished in the HPLC chromatogram, and only a fraction of the sample mass under HPLC was required for UHPLC. Yet the molecular weights determined by the MALS detector for both techniques are very close. The UHPLC upgrade path effectively future-proofs SEC-MALS instrumentation for the transition to UHPLC.

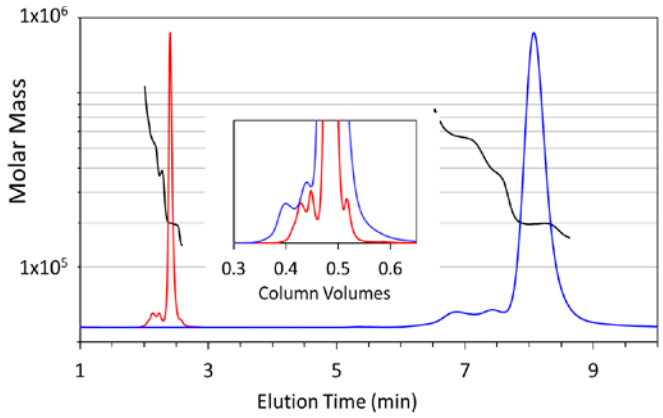


Figure 9. Comparison of an HPLC protein separation (200 µg injection, blue trace) measured with the as-shipped TREOS II, and a UHPLC measurement (30 µg injection, red trace) measured with the TREOS II after switching the optical module to the UHPLC-compatible, microflow cell optical module. Molar masses of the monomer and aggregates are essentially identical in both. Inset: chromatograms with elution volume normalized to column volume.

Summary

The miniDAWN TREOS II exhibits a major leap forward in performance, productivity and versatility over other compact MALS detectors. In particular, its fully modular design combined with powerful software features make SEC-MALS easy, reliable and routine, well suited for mission-critical tasks that cannot tolerate extended downtime or operational complexity. Absolute macromolecular characterization, essential for cutting-edge biopharmaceutical and polymer research and development, is ready for everyday use in demanding environments.

References

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